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Dept. of Ecology Water Quality Program
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Toxicity Testing to Establish the Environmental Safety of Proposed Ballast Water Biocides

Purpose of Ballast Water Biocide Toxicity Testing Procedures

The Washington Department of Fish and Wildlife (WDFW) has been tasked by the state legislature with setting standards for ballast water treatment and coordinating a two panel (science advisory panel and maritime advisory panel) review process for technologies proposed for meeting that standard. The science advisory panel will provide recommendations regarding the ability of each technology to meet the Washington state interim ballast water discharge standard and the adequacy of the proposed study plan. The maritime advisory panel will provide recommendations regarding the ability of each technology to meet the practical needs of the maritime industry, including safety, practicality, and cost-effectiveness. Both panels will determine if any ballast water treatment system should be considered for "best available technology" status. Interim approvals will last five years. Any problems found during the five years must be fixed or approval will be withdrawn. New approval requirements may be applied after five years. The Department of Ecology will be advising WDFW on environmental safety issues and setting conditions on the discharge of biocide-treated ballast water. This document describes the testing needed by the Department of Ecology in order to advise WDFW on applications for treatment system interim approval.

Authority:

> **WAC 220-77-095 Interim ballast water discharge standard approval process.**
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> (2)(d) Criteria for review. Applications for interim approval of a ballast water treatment system shall be evaluated on the completeness of the following:
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> (ii) Documentation stating that the residual concentrations of any primary treatment chemicals or chemicals that occur as by-products of the treatment meet all applicable regulatory requirements.
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> (v) The discharge from a technology must be environmentally sound and in compliance with existing water quality discharge laws.
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> (e) Each proposed technology must include a detailed study plan that:
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> (i) Is organized according to a department-approved standardized format.
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> (3)(b) Systems approved under the interim approval process shall be considered to meet all ballast water treatment requirements promulgated by the department for a period of five years. In the event subsequent work reveals adverse effects on ecology or human health, approval of the system will be withdrawn unless the treatment system can be repaired to address the system's inadequacies.

Which Ballast Water Biocides Need Toxicity Testing?

All ballast water biocides will need some form of testing in order to determine their effectiveness in meeting WAC 220-77-095 which requires inactivation or removal of ninety-five percent of zooplankton organisms and ninety-nine percent of phytoplankton and bacterial organisms. This testing to determine efficacy might include toxicity testing. Proposed ballast water biocides may also need toxicity testing in order to protect receiving waters from toxic effects due to ballast water discharges. Toxicity testing to assess potential impacts on receiving water is the responsibility of the applicant for interim approval of a ballast water treatment system that utilizes a biocide. This toxicity testing may need to be separate from the toxicity testing done to determine the efficacy of the biocide. Efficacy testing focuses on resistant organisms while environmental safety testing focuses on sensitive organisms.

Chemical ballast water biocides accomplish their intended purpose through toxicity. Biocide toxicity is beneficial as long as it does not act against nontarget species or outside of its intended location. Because ballast water might contain both unwanted nonindigenous species and native species which could be disease carriers, there is no such thing as a nontarget species for a ballast water biocide. However, if the ballast water is excessively toxic at the time of discharge into the environment, receiving water organisms will suffer unacceptable harm. Toxicity testing is needed to determine conditions when the potential for the biocide to harm receiving water organisms is unacceptable. Solely physical ballast water treatment methods such as filtration, centrifugation, ultraviolet irradiation, or oxygen stripping are assumed to not need toxicity testing.

Biocide Toxicity Tests

The testing should include a 96-hour acute toxicity test with silverside minnows (*Menidia beryllina* in EPA-821-R-02-012), a 48-hour acute toxicity test with a mysid (*Mysidopsis bahia* in EPA-821-R-02-012), and a 48-hour bivalve embryo-larval survival and development test (EPA/600/R-95-136). Ballast water discharges are short enough in

duration to not need testing with the 7-day survival and growth tests unless there is the possibility that more than one ship during the same week will be discharging ballast water containing the same biocide at the same pier or at an adjacent pier in the same port. If the 7-day chronic tests are determined to be necessary due to multiple discharges at the same location in the same week, then the 7-day survival and growth tests in EPA-821-R-02-014 using silverside minnows (*Menidia beryllina*) and a mysid (*Mysidopsis bahia*) must be conducted. If the 7-day chronic tests are conducted, the acute tests with the same species are unnecessary. The bivalve test must still be conducted even if the 7-day chronic tests are used. The results from the most sensitive test will be used in making decisions. The most sensitive test will usually be the bivalve test but the fish and mysid still need to be tested to make sure that the biocide does not have some special toxicity to fish or crustaceans. The 7-day chronic tests with the west coast topsmelt (*Atherinops affinis*) or mysid (*Holmesimysis costata*) in EPA/600/R-95-136 are acceptable substitutes for the east coast test organisms. The echinoderm embryo-larval survival and development test in EPA/600/R-95-136 is an acceptable substitute for the bivalve development test and may be easier in the time series testing described below for Biocide Toxicity Testing to Demonstrate Zero Toxicity at Ballast Water Discharge.

Test solutions are renewed at 48 hours in a 96-hour acute test and daily for the 7-day chronic tests (except for *Holmesimysis*). Test solutions should be renewed with the original biocide solution prepared at the start of the test. The biocide solution should be stored in the dark at 8 ± 1ºC with minimal headspace. The storage container should be made out of a substance to which the biocide would not adsorb or react.

Test reports must meet the reporting requirements in the EPA toxicity testing manuals and describe test conditions such as test chamber size, test solution volume, temperature, dilution water source, exact test start time, exact test end time, etc. Test reports must contain a readable copy of all hand-written bench sheets. The bench sheets must include both the toxicological and water chemistry data for both the biocide tests and reference toxicant tests. The bench sheets must contain actual counts (not percentages) in order to be acceptable. Start counts must be clearly recorded on the bench sheet. The test report must include computer printouts of test data and statistical analyses. Test organism source, age, and unusual conditions (lethargy, hyperactivity, spots or filaments, discoloration, excessive ventilation, etc.) must be reported. The report must contain a description and justification of any aeration or pH control/modification used during the test. Each test report must contain a section where all deviations from test protocols must be accurately listed or the absence of such deviations noted.

It might not be possible to measure biocide concentrations during testing. A toxicity testing lab may not be capable of doing so and an offsite lab may not be acceptable because of biocide degradation during sample shipment. However, if the biocide concentration can be analyzed reliably in the toxicity testing lab, then it should be done in one test chamber (or a surrogate) at each test concentration at the beginning and end of the test. If it can't be measured reliably, then the concentrations should be assumed to be as prepared.

The toxicity tests mentioned above cannot be done reasonably on ships. The tests require special skills and facilities which are lacking on the majority of vessels. The tests take too long to conduct for a ship operator needing to discharge ballast. The goal of the testing described in this document is not routine monitoring of ballast water toxicity just prior to discharge but to establish in other ways how to keep ballast water toxicity from being an environmental threat. If any toxicity test ever becomes established to our satisfaction as being reliable and convenient enough for routine use on a ship, then this document will be revised to allow for its use instead of the other testing described here.

Laboratory toxicity testing was chosen because not all applicants will have access to a ship for an onboard study. However, *in situ* toxicity testing done inside ballast water tanks will be the most realistic testing possible. If an applicant has *in situ* toxicity testing data from an onboard study done with organisms of comparable sensitivity to the species listed above, then the study report and toxicity test data may be submitted instead of doing laboratory testing. The report must contain data on relative sensitivity. The plan for an onboard study may be submitted for consultation in advance if that would be helpful in meeting requirements.

Biocide Toxicity Testing to Demonstrate Zero Toxicity at Ballast Water Discharge

Some biocides will not need to maintain a residual toxicity until discharge in order to meet the requirement in WAC 220-77-095. After repeated treatment with an effective biocide, a ship may no longer need to maintain toxicity until ballast water discharge in order to inactivate or remove ninety-five percent of zooplankton organisms and ninety-nine percent of phytoplankton and bacterial organisms. Circumstances which would aid in reaching this situation include the removal of suspended particles (including multicellular organisms) from water prior to entry into ballast tanks, the dosing of a highly toxic and highly soluble biocide into water as it is being pumped into ballast tanks, and good cleaning and maintenance of ballast tanks. All ballast water treatment systems utilizing biocides should eventually get to the point where residual toxicity in the ballast water discharge is no longer necessary. Not only will this mean that

environmental risk will be lower, but the ship will not need to measure biocide concentration in the ballast water at discharge in order to verify meeting the target discharge concentration and will simply need to note the time elapsed from treatment to discharge. However, a ship may not always be able to wait for the entire time period needed for biocide toxicity to disappear before discharging ballast water. Biocide treatment may need to be conducted during a voyage if a planned mid-ocean exchange is not possible or if there are doubts about the effectiveness of the original biocide treatment of the ship's ballast water. The ability to set a maximum allowable toxicity level for ballast water is important for these reasons.

The toxicity of a biocide in ballast water must begin as highly toxic and gradually become nontoxic due to volatilization, reaction, or degradation or it will either be ineffective or too risky to discharge. Toxicity testing will be used to demonstrate this process and to verify that no other toxic compounds have formed which might persist longer than the biocide. The first step is to determine a maximum biocide concentration which might be used. The next step is to add the biocide to seawater up to that concentration and hold under conditions that are as close as possible to actual use in a ballast tank. These storage conditions would include 8 ± 1ºC in the dark. The surface area to volume ratio of the storage container should be as small as practical in order to resemble a ballast tank. The storage container should be made out of a substance to which the biocide would not adsorb or react.

The biocide toxicity tests listed above must be conducted on samples drawn from the storage container every four hours over a 24-hour time period chosen so that the estimated time for the disappearance of toxicity is near the end of the period. (The ages of biocide treated water used in the 2nd paragraph below begin at the time chosen as the beginning of the last 24 hours of biocide toxicity and are not the time of treated water preparation.) A pretest may be needed to estimate the time for disappearance of biocide toxicity. The pretest may also determine the most sensitive species so only that species needs tested with all of the samples. Concentration series are not required since a time series is the testing goal.

An alternate approach is to prepare separate volumes of biocide treated water at 4-hour intervals and subsequently initiate all of the toxicity tests at the same time on samples drawn from each volume. Beginning all of the toxicity tests at the same time would be easier than initiating new tests every four hours. This alternative would require a fair amount of refrigerated storage space and special care in preparing the several volumes of treated water, but would allow daily maintenance activities to be conducted from the beginning of the study at equal intervals.

The most efficient method for accomplishing this task is to setup a schedule similar to the following: Prepare biocide-treated water at 4:00, 8:00, 12:00, 16:00, and 20:00 on one day. At 16:00 on a subsequent day determined to be the beginning of the last 24 hours of toxicity, use the treated water from 4:00, 8:00, 12:00, and 16:00 to start tests for treated water of ages 0, 4, 8, and 12 hours. Start tests at 12:00 on the next day using the treated water prepared at 12:00, 16:00, and 20:00 to represent water of ages 16, 20, and 24 hours, and then do the daily renewals, etc. as soon as possible for the tests begun on the first day. Conduct daily renewals, etc. at 14:00 for the duration of the testing. (Not all tests are required to have test solutions renewed daily; see the EPA test method or Ecology Publication WQ-R-95-80 for details.)

The testing schedule above, involving samples drawn from a storage container every four hours over a 24-hour time period, may be modified as long as at least one of the samples is toxic and a subsequent sample is nontoxic. Caution in setting an alternative testing schedule should be exercised so that the minimum time needed before ballast water discharge is not overestimated and ship operators are not needlessly inconvenienced. Repeating a study can be as expensive as investing extra effort into the first attempt.

The results of the ballast water biocide time series toxicity testing must be submitted to the Department of Ecology Water Quality Program's Whole Effluent Toxicity (WET) Coordinator for determination of the time to zero toxicity. The earliest sample to have no statistically significant toxicity relative to a control will be the indicator of the minimum time needed before discharge.

Biocide Toxicity Testing to Maintain Moderate Residual Toxicity at Ballast Water Discharge

Moderate toxicity at the time of ballast water discharge may be necessary for some biocides to accomplish the intended purpose of preventing the introduction of potential disease organisms. Under some circumstances, ballast water toxicity might need to persist until discharge in order to meet state standards for the reduction of bacteria. The complete elimination of bacteria in ballast tanks is impossible and those remaining can produce a population rebound when toxicity goes away. The rebound will be encouraged if the dead bodies of multicellular animals killed by the biocide are available as food for the surviving bacteria. This situation would favor the bacteria that can feed on animal tissue and that would therefore more likely be pathogens of fish or other important marine organisms. The same concerns would apply to fungal pathogens as well.

Because biocides are chemicals selected for their toxicity, biocides tend to have steep concentration-response relationships. A steep concentration-response relationship means that the difference between a concentration that is toxic and one that is nontoxic will be small. Toxicity testing can be used to determine the smallest difference that exists between the toxic and nontoxic concentrations of a biocide so that a target discharge concentration can be set such that ballast water toxicity will be eliminated very quickly after discharge. Biocides lacking a steep concentration-response relationship should not be proposed and are not likely to be found acceptable for discharge with residual toxicity. Because the growth measurement in the 7-day tests with fish or mysids usually does not yield a steep concentration-response relationship, it cannot be used be used in setting the maximum allowable residual toxicity for a ballast water biocide as described below. However, the development endpoint in the bivalve test is usually more sensitive than fish or mysid growth and generates steep concentration-response relationships.

The toxicity testing to determine the maximum allowable residual toxicity will need to focus test concentrations around the toxic threshold for lethality to fish or mysids or for combined survival and development in bivalves. A range-finding test may need to be done first in order to find the general vicinity of the toxic threshold. The tests must have a series of at least five concentrations based on a dilution factor of ≥ 0.5 and the toxic threshold (LC_{50} or NOEC/LOEC) should ideally be in the middle to upper part of the lower half of the concentration series where spacing between concentrations is small. A few partial responses are greatly desired since they will allow calculation of an LC_{25}. At least four replicates must be run at each concentration for every test. If the range-finding testing clearly determines a most sensitive species, then definitive testing may be conducted using only that species.

The results of the ballast water biocide definitive toxicity testing must be submitted to the Department of Ecology Water Quality Program's Whole Effluent Toxicity (WET) Coordinator for calculation of the toxic threshold and determination of the target discharge concentration. The results of range-finding tests must also be included if they were used to determine the most sensitive species prior to definitive testing. The report must also propose an onboard method for accurately measuring either biocide concentration or a meaningful surrogate in order to verify that the biocide is near the target discharge concentration just prior to beginning ballast water discharge. Candidates for the target discharge concentration include in order of preference:

1. A target discharge concentration will be set at two times the LC_{50} as long as the LC_{50} is no more than three times the LC_{25}. If it does not seem likely from the data that two times the LC_{50} would produce a complete effect, then the target discharge concentration will be set at three times the LC_{50} as long as the LC_{50} is no more than two times the LC_{25}. If the bivalve test is the most sensitive, the EC_{50} and EC_{25} for combined survival and development will be used as described for the LC_{50} and LC_{25}.
2. A target discharge concentration will be set at three times the LOEC if a concentration at or between two to four times the LOEC caused complete mortality.

Unless the conditions for one of the candidate methods above can be met in the determination of the target discharge concentration, the testing needs repeated with an improved concentration series. If the lowest concentration tested shows a statistically significant effect relative to the control, then the testing needs repeated with an improved concentration series.

Using a Combination of Testing Strategies May Make Sense

Performing both of the testing strategies described above may make good sense. Testing to demonstrate zero biocide toxicity at ballast water discharge is preferable if the biocide performance is good enough. However, a ship may not always be able to wait for the entire time period needed for biocide toxicity to disappear before discharging ballast water. Biocide treatment may need to be conducted during a voyage if a planned mid-ocean exchange is not possible or if there are doubts about the effectiveness of the original biocide treatment of the ship's ballast water. Testing to determine a biocide concentration that is moderately toxic but considered safe to discharge will give ships some potentially useful flexibility in discharge timing.

Toxicity Testing to Verify Biocide Neutralization

The ballast water biocide that would be most protective of the environment and convenient for ship operators would be one that would stay toxic during the voyage and could be neutralized just prior to discharge. For example, a toxic oxidant could be easily neutralized by a reducing agent. The neutralizing chemical would also have toxicity that would likely be less than the toxicity of the biocide that it neutralizes. After a demonstration that the neutralizing chemical can effectively eliminate biocide toxicity without any toxic reaction products, toxicity testing will focus on determining the

safety margin for the neutralizing chemical so that care can be given to prevent the toxic threshold of the neutralizing chemical from ever being reached.

The safety margin is the difference between the maximum intended use concentration of the neutralizing chemical, the intended discharge concentration after reaction with the biocide, and the threshold of toxicity to the most sensitive species tested. The concentration series for each toxicity test must include the maximum intended use and discharge concentrations for the chemical and its toxic threshold. A range-finding test may be necessary to estimate the toxic threshold before performing a definitive toxicity test to determine the safety margin. If the range-finding tests clearly identify a most sensitive species from the species listed above, then definitive testing may focus solely on that test species.

The best analytical methods for determining toxic thresholds are point estimation techniques because they can interpolate between concentrations and avoid overestimating the safety margin by using the LOEC or underestimating the safety margin by using the NOEC. The 25% effect level is used by EPA as an estimate of the toxic threshold. The IC_{25} should be used for growth and the EC_{25} for the survival and development endpoints. If an EC_{25} cannot be calculated, then the MATC (geometric mean of the NOEC and LOEC) should be used or the test should be repeated with more concentrations around the anticipated toxic threshold. No test should have less than four replicates.

Once the toxicity testing has been done and the safety margin determined, then a plan must be submitted describing how the neutralizing chemical will be used in order to be both effective and maintain the safety margin. The plan must address how monitoring will maintain the safety margin during routine use. Chemicals that are used for neutralizing other chemicals are usually also neutralized during the process. If the neutralizing chemical is properly dosed, there will be little or no chemical carry-over, but the method for maintaining proper dosing relative to varying biocide concentrations must be described. Recommendations will be based on the confidence generated by the plan that the safety margin for the neutralizing chemical will always be maintained.

If the toxic threshold is above the maximum intended use concentration, then monitoring of the chemical dosing relative to the ballast water discharge volume should be adequate to demonstrate maintenance of the safety margin. If the toxic threshold is between the maximum intended use concentration and the intended discharge concentration, then a removal rate for the chemical needs to be determined at different biocide concentrations and that information used along with monitoring of chemical dosing relative to both the biocide concentration and the ballast water discharge volume in order to demonstrate maintenance of the safety margin. If the discharge concentration of the neutralizing chemical can be reliably measured onboard on a routine basis, then monitoring of the discharge concentration can be used as an alternative method for demonstrating maintenance of the safety margin when the toxic threshold is between the intended use and discharge concentrations. If the toxic threshold is below the intended discharge concentration, then the neutralizing chemical must not be used. If the toxic threshold for lethality is less than five times the intended discharge concentration, then the safety margin is considered to be too narrow and the neutralizing chemical must not be used.

Report to the WDFW Aquatic Nuisance Species Coordinator

The WET Coordinator will review the test results and determine a target discharge concentration or a minimum time before it is acceptable to discharge. The WET Coordinator will then make a report to the WDFW Aquatic Nuisance Species Coordinator for inclusion in the interim ballast water discharge standard approval process in WAC 220-77-095. The report will also note any concerns the Department of Ecology may have about the biocide's persistence, ability to bioaccumulate, or potential hazards to human health.

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